



IDENTIFICATION AND CLONING OF A MYCOBACTERIAL ANTIGEN CORRESPONDING TO A HEPARIN-BINDING HAEMAGGLUTININ

invent
AA₁ →

5 The invention relates to peptide sequences enabling mycobacteria to adhere to host cells, in particular to epithelial cells. More particularly, the invention relates to a mycobacterial heparin-binding haemagglutinin (HBHA) type antigen obtained from *Mycobacterium bovis* BCG or *Mycobacterium tuberculosis*. The invention also relates to a recombinant peptide sequence enabling mycobacteria to adhere to host cells. In particular, the invention relates to the expression product of an *Escherichia coli* strain transformed with a nucleotide
10 sequence coding for a protein enabling mycobacteria to adhere to host cells. These polypeptides can be used in immunogenic compositions, to prepare vaccines against mycobacterial infections, and for serological diagnosis of mycobacterial infections.

The invention also relates to a nucleotide sequence coding for a peptide
15 sequence enabling mycobacteria to adhere to host cells, and in particular a nucleotide sequence coding for a mycobacterial heparin-binding haemagglutinin (HBHA) type antigen. The invention also relates to recombinant vectors comprising said nucleotide sequence and to the use of these vectors in producing recombinant host cells which can be used in therapy, in particular in anti-cancer
20 therapy.

Mycobacteria are among the most important pathogenic micro-organisms which cause disease in both man and in animals. Mycobacterial infections are still among the main causes of death in the world. Human tuberculosis, caused by *Mycobacterium tuberculosis*, by itself leads to approximately 3 million deaths per
25 annum (1, 2). *Mycobacterium bovis* causes tuberculosis in cattle, but it is also highly virulent in man. Leprosy, caused by *Mycobacterium leprae*, remains a major unresolved health problem in developing countries (3).

Infections by members of the *Mycobacterium avium intracellulare* complex cause disease in birds and in pigs and are among the most frequent
30 opportunistic infections found in patients suffering from acquired

immunodeficiency syndrome (AIDS) (4, 5). Further, the recent dramatic re-appearance of tuberculosis in developed countries and the appearance and propagation of drug resistant *M. tuberculosis* strains (6) underline the difficulty of controlling mycobacterial diseases.

5 Molecular characterisation of the various steps in the pathogenesis of mycobacterial diseases is fundamental to the development of optimised and rational therapeutic and prophylactic approaches to such diseases. The virulence carriers are often good antigens which can be used as candidates for vaccines. Despite the importance of mycobacterial infections, little is known about the basic
10 molecular mechanisms involved in their pathogenesis (7).

One of the initial and crucial events in bacterial pathogenesis is adhesion of the micro-organism to its target cells. Mycobacteria exhibit tropism for pulmonary macrophages (8). However, since such micro-organisms are readily transmitted by aerosol, the first structures in the host which they encounter during
15 infection are those of the respiratory epithelium. As a result, interactions with epithelial cells or with the extracellular matrix (ECM) during the initial and subsequent steps of pathogenesis can be important (9), although they have not yet been studied to any great extent.

Within the context of the present invention, the inventors have obtained a
20 novel mycobacterial antigen involved in adhesion of mycobacteria to epithelial type host cells. It is a 28 kDa heparin binding haemagglutinin (HBHA) which has been obtained from culture supernatants prepared from cell walls of *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis*. Immunoblot analysis using polyclonal and monoclonal antibodies have indicated that HBHA
25 differs from proteins of the antigen 85 complex and represents a novel antigen. From this basic protein, the inventors have been able to evaluate and propose the development of a series of polypeptides which can be used for diagnosis, therapy and prophylaxis.

The invention thus provides a peptide sequence enabling mycobacteria to
30 adhere to host cells, in particular epithelial cells. More particularly, the peptide

sequence of the invention is characterized in that it is a mycobacterial heparin-binding haemagglutinin (HBHA) type antigen, in particular an antigen obtained from *Mycobacterium bovis* BCG or *Mycobacterium tuberculosis*.

1 In a preferred embodiment of the present invention, the peptide sequence is characterized in that it comprises the sequence corresponding to the sequence shown in Figure 10, or any variant of that sequence which enables mycobacteria to adhere to host cells and obtained by addition, substitution or deletion of one or more amino acids of the sequence of Figure 10.

Throughout the text, the term "polypeptide sequence" or "polypeptide" represents all or part of the sequence of Figure 10, which itself represents the DNA and the HBHA protein as it is. The term "protein" designates the modified or non modified polypeptide sequence.

2 Preferably, the invention more particularly provides a peptide sequence comprising a region involved in interactions with sulphated glycoconjugates and in heparin binding. This peptide sequence is as follows:

KKAAPAKKAAPAKKAAPAKKAAAKKAPAKKAAAKKVTQK

The invention thus concerns a peptide sequence comprising the C-terminal portion of the sequence of Figure 10 and more particularly the sequences comprising approximately the last 30 to 50 amino acids of the C-terminal portion of the sequence of Figure 10. This region of the sequence of Figure 10 is involved in the interaction of the protein with heparin, although a shorter sequence, in particular of about 10 to 20 amino acids, may be sufficient.

The inventors have also expressed the nucleotide sequence coding for the peptide sequence of the invention in *E. coli*. The polypeptide obtained has a lower molecular weight than that of the purified protein from *M. bovis* BCG or *M. tuberculosis*. These differences are the result of post-translational modifications which do not occur in *E. coli*.

The invention thus also provides a recombinant peptide sequence, characterized in that it enables mycobacteria to adhere to host cells. More particularly, the recombinant sequence of the present invention is the expression

product of a nucleotide sequence coding for a peptide sequence enabling mycobacteria to adhere to host cells, in particular an antigenic sequence obtained from *M. bovis* BCG or *M. tuberculosis*, said recombinant sequence being, for example, the expression product of an *E. coli* strain transformed with a suitable
5 nucleotide sequence.

The invention also relates to the use of one of the peptide sequences described above, whether recombinant or non recombinant, and more particularly in its native form for serological diagnosis of the presence of mycobacteria. The invention also provides an immunogen composition characterized in that it
10 comprises one of the peptide sequences described above and to the use of that peptide sequence to prepare vaccines against mycobacterial infections, particularly infections caused by *M. bovis* or *M. tuberculosis*.

The inventors have also discovered that adhesion of mycobacteria to epithelial cells can be specifically inhibited by sulphated glucides. The invention
15 thus concerns the use of a sulphated glucide to inhibit adhesion of mycobacteria to epithelial cells. Sulphated glucides of particular interest include heparin, chondroitin sulphate and dextran sulphate as well as their synthetic derivatives.

The inventors have also isolated the whole of the gene coding for HBHA from the DNA of *M. bovis* BCG. The invention thus provides a nucleotide
20 sequence, characterized in that it codes for a peptide sequence enabling mycobacteria to adhere to host cells. More particularly, the nucleotide sequence of the invention codes for a mycobacterial heparin-binding haemagglutinin (HBHA) type antigen, in particular the peptide sequence of Figure 10 or any portion of that peptide sequence enabling mycobacteria to adhere to host cells and
25 obtained by addition, substitution or deletion of one or more amino acids from said peptide sequence.

The invention also provides a recombinant host cell, characterized in that it comprises one of the nucleotide sequences described above in its genome. In one preferred embodiment of the invention, the recombinant host cell is BCG, but not

exclusively, for which expression vectors directly usable for developing recombinant BCG for use in man or animal have been developed.

BCG is used in therapy, more particularly in anti-cancer therapy, in particular against superficial cancers of the bladder. In this type of therapeutic application, a correlation between the adhesion ability of the BCG and its anti-tumoral power appears to exist. Within the context of the present invention, identification of the HBHA and cloning of its gene renders possible an increase in adhesion capacity via overexpression of the gene coding for HBHA.

1 The present invention will now be described with reference to the following Figures:

- Figure 1 shows the effect of sulphated and non sulphated glucides on mycobacterial adhesion to CHO cells and to macrophages;
- Figure 2 shows data demonstrating purification of a heparin binding protein of *M. bovis* BCG;
- 15 • Figure 3 shows a comparison of the heparin binding protein of *M. bovis* with the antigen 85 complex;
- Figure 4 shows the effect of sulphated glucides and non sulphated glucides on haemagglutination induced by HBHA;
- Figure 5 shows the inhibition of adhesion of BCG to CHO cells by anti-HBHA antibodies;
- 20 • Figure 6 shows immunoblot analyses carried out with tuberculous anti-sera;
- Figure 7 shows the nucleotide sequence and the amino acid sequence of a fragment of HBHA deduced from a PCR fragment of chromosomal BCG DNA;
- 25 • Figure 8 shows a Southern blot analysis of chromosomal BCG DNA;
- Figure 9 shows the sequencing strategy for the gene coding for HBHA;
- Figure 10 shows the DNA sequence of the BCG gene coding for HBHA;
- Figure 11 shows a polyacrylamide gel electrophoresis and immunoblot analysis of the expression of HBHA in *E. coli*.

DETAILED DESCRIPTION OF THE INVENTION

Inhibition of mycobacterial adhesion to epithelial cells by sulphated glucides

The invention concerns the use of sulphated glucides to inhibit adhesion of mycobacteria to epithelial cells. To demonstrate that mycobacteria can adhere via sulphated glucides, the inventors tested whether soluble sulphated polysaccharides were capable of reducing the adhesion of *M. bovis* BCG to epithelial cells.

M. bovis BCG (strain 1173P2, OMS, Stockholm, Sweden, passes 3 to 8) in exponential growth were labelled by cultivating the mycobacteria for three days in Sauton medium containing 5 $\mu\text{Ci/ml}$ of $[6\text{-}^3\text{H}]\text{uracil}$ (New England Nuclear, 24 Ci/mmmole). The mycobacteria were then harvested by centrifugation ($3000 \times g$ for 5 minutes), washed twice with a Dulbecco saline phosphate buffer (DPBS) and taken up into suspension in RPMI 1640 culture medium containing 300 mg/l of L-glutamine (GIBCO), free of foetal calf serum (RPMI).

INS
FH
The day before the adhesion test, the wells of 24-well tissue culture plates (Nunc, Nunc, Denmark) were inoculated with 10^5 extemporaneously cultivated cells from the ovaries of Chinese hamsters (CHO) (see Figures 1A and 1B, grey bars) or J774A.1 macrophages (ATCC TIB67) (see Figure 1B, black bars) taken up into suspension in 2 ml of RPMI with an added 10% (v/v) of decomplexed foetal calf serum (RPMI-FCS). Just before the test, the cells were washed three times with 2 ml of RPMI, and 1 ml of the mycobacterial suspension in RPMI was added to each well to obtain an infection multiplicity of 10 bacteria per eukaryote cell.

The adhesion test was carried out in the presence of increasing concentrations of D(+)-galactose (Sigma) (see Figure 1A, black circles) or heparin from pork intestinal mucosa (M_r 6 kDa, Sigma) (see Figure 1A, white circles), or with 20 $\mu\text{g/ml}$ of the indicated glucides (Sigma) (see Figure 1B). After 6 hours of incubation at 37°C in an atmosphere containing 5% of CO_2 , the cells were washed three times in 2 ml of DPBS, and finally lysed by adding 1 ml of distilled water containing 0.1% (w/v) of sodium deoxychlorate. The radioactivity associated with the cellular lysates was counted using a liquid scintillation counter (Beckman, model LS 6000SC). Residual adhesion was expressed as a percentage of the radioactive

counts per minute with respect to the counts obtained in the absence of glucide. The data shown in Figures 1A and 1B represent the averages of four experiments, and show the error bars.

As shown in Figure 1A, low concentrations of heparin substantially inhibited adhesion to CHO cells of BCG labelled with [³H]uracil, while up to 100 µg/ml of galactose had no significant effect. Dextran sulphate, fucoidane and chondroitin sulphate could reduce adhesion, but no significant inhibition was observed with non sulphated mannose or dextran, even at the highest concentrations tested (1 mg/ml).

These results lead to the proposal that mycobacteria have a major sulphated glucide binding adhesin on their surface. However, as adhesion inhibition did not exceed 70%, other components are very probably involved in this process. Interestingly, interactions of BCG with J774A.1 macrophages were not affected by sulphated glucides, nor by non sulphated sugars (Figure 1B), even at concentrations of up to 1 mg/ml (results not shown). This is in agreement with previous reports naming receptors of the CR1, CR3 and CR4 complement as mycobacterial ligands on the surface of sanguine monocytes and alveolar macrophages (10-12).

Purification of a heparin binding protein present in *M. bovis* BCG and *M. tuberculosis*

The invention concerns a peptide sequence enabling mycobacteria to adhere to host cells. Inhibition of adhesion of BCG to epithelial cells by sulphated sugars would already suggest that mycobacteria express an adhesin which interacts with sulphated glycoconjugates, such as sulphated glycoproteins or glycolipids present on the surface of the host cells. To identify the adhesins in question, BCG culture supernatants were fractionated by chromatography on heparin-sepharose.

M. bovis BCG were cultivated at 37°C in static cultures in 175 cm³ Roux flasks (Falcon, Becton-Dickinson) containing approximately 150 ml of Sauton medium. In the stationary phase, the cultures were centrifuged (10000 x g for 20

minutes), and 500 ml of supernatant was loaded onto a heparin-sepharose CL-6B column (1 x 5 cm) (Pharmacia) equilibrated with DPBS. The column was then washed with 100 ml of DPBS, and the retained molecules were eluted by a linear gradient of NaCl, 0-500 mM, in 100 ml of DPBS. The flow rate during all of the steps was kept at 1.5 ml/min, and the absorbance of the eluent, which was harvested in 1 ml fractions, was continuously monitored at 280 nm. These fractions were analysed by SDS-PAGE (13) using a 12% gel followed by staining with Coomassie Brilliant Blue R-250.

The fractions analysed after the start of the gradient are shown in tracks 1-8 of Figure 2. The right hand tracks show the heparin binding protein purified from a preparation of *M. bovis* BCG cell walls. The size of the markers M_r , expressed in kDa, are given in the right hand margin.

As shown in Figure 2, a single protein of 28 kDa was eluted at approximately 350 mM of NaCl. The interaction between this protein and heparin was dependent on the sugar sulphatation as that could also be purified using dextran sulphate beads, but not with dextran beads (not shown). The same heparin binding protein was also purified from *M. tuberculosis* culture filtrates.

The production of two similar heparin binding proteins from two different mycobacterial strains confirms that proteins of this type are present in the majority of pathogenic mycobacterial strains. Homology percentages of less than 100% could lead to structural differences, although the fundamental properties of these proteins are not affected. The invention thus also concerns heparin binding proteins with a structure related to the isolated protein and which can be obtained using its properties of adhesion to sulphated glucides. These proteins can be distinguished from the protein of the invention by additions, substitutions and deletions of amino acids without that substantially affecting their properties of adhesion to epithelial cells.

Localisation of the heparin binding mycobacterial protein on the cell surface.

In order to determine whether this protein is a strictly secreted protein or if it could be associated with the mycobacterial surface, cell wall fractions were prepared from BCG, then chromatographed on heparin-sepharose.

To prepare the cell walls, mycobacteria were cultivated in 2 litres of Sauton medium or in Long synthetic medium (Quality Biological Inc., Gaithersburg, MD) for 12 to 14 days. The bacteria were then harvested by centrifugation, washed once with DPBS containing 0.05% (v/v) of Tween 80 (DPBS/Tw), taken up into suspension in 100 ml of DPBS/Tw and heated to 80°C for 1 hour. The bacteria were centrifuged at 13000 x g for 20 minutes, washed with DPBS/Tw, taken up into suspension in 25 ml of DPBS/Tw containing 5 nM of protease inhibitor AEBSF, RNase A and DNase I, sonically lysed intermittently for 25 minutes, then centrifuged at 13000 x g for 20 minutes. This step was repeated and the supernatants were combined and centrifuged at 34000 x g for 3 hours at 4°C. The residue was discarded and the final supernatant was diluted 1:2 in DPBS and chromatographed on CL-6B heparin-sepharose.

As shown in Figure 2, the 28 kDa heparin binding protein was also present in these preparations, confirming that it was associated with the surface. It was also present in cell wall fractions of *M. tuberculosis* and in tuberculine preparations (purified antigenic proteinic derivative for cutireaction) derived from *M. tuberculosis*.

Haemagglutination activity of the mycobacterial heparin binding protein

The capacity of bacterial adhesins to agglutinate red globules is often used as a model to study microbial binding such as that of lectins to the receptor in eukaryotic cells. The inventors thus tested the purified heparin binding protein for its capacity to agglutinate erythrocytes.

The haemagglutination activity of the purified heparin binding protein in culture supernatants was measured in U microtitration plates (Falcon) in the absence or in the presence of heparin (Figure 4, black squares) from pork intestinal mucosa, dextran sulphate (Figure 4, black circles) or dextran (figure 4, white circles) in concentrations of 0 to 50 µg/ml. Standard tests contained 70 µl

of a fresh suspension of rabbit erythrocytes prepared in DPBS and 70 μ l of purified HBHA corresponding to 1 μ g of protein. The amount of haemagglutination was read after 5 hours of incubation at room temperature. The data represent the averages over four experiments, and the error bars are shown.

Less than 0.1 μ g of purified protein was able to induce haemagglutination of rabbit erythrocytes, but not of human, sheep, goose or chicken erythrocytes (data not shown). For this reason, the inventors designated this protein as being the heparin binding haemagglutinin (HBHA).

Haemagglutination induced by HBHA was inhibited by heparin or by dextran sulphate, but not by dextran (see Figure 4), which is similar to the results obtained in the tests for adhesion of mycobacteria to CHO cells. The haemagglutination activity was heat sensitive in that incubation of the protein for 60 minutes at 80°C destroyed haemagglutination.

Inhibition of haemagglutination activities and adhesion originating from mediation by HBHA, by specific polyclonal and monoclonal antibodies

Since mycobacterial adhesion to epithelial cells is inhibited by sulphated glucides, and a 28 kDa protein was purified by heparin-sepharose chromatography, it was important to establish whether mycobacterial adhesion originated from mediation by HBHA.

As a result, anti-HBHA rat polyclonal antibodies were prepared as follows: 200 μ g of purified BCG HBHA underwent preparative electrophoresis on a 15% polyacrylamide gel in the presence of SDS, then electro-transferred to a nitrocellulose membrane. After rapid staining with xylidene red, the bands corresponding to HBHA were excised with care, cut into small squares and briefly sonically lysed in 1.5 ml of sterile PBS. After adding 1 ml of a monophosphoryl-lipid A solution (MPL + TDM System Adjuvant, Sigma Chemical Co., St. Louis, MO) prepared in accordance with the manufacturer's recommendations, two Fischer rats were each immunised with 1 ml of the antigen suspension. For each immunisation, 400 μ l was administered intraperitoneally and two times 300 μ l

subcutaneously. The animals received repeat immunisations in the same manner with the same quantity of antigen one month later, and the serum was recovered 3 weeks after that repetition.

125
F8
5
The BCG was labelled with [6-³H]uracil as described above. Approximately 4×10^6 radiolabelled BCG were taken up into suspension in 1 ml of PBS and pre-incubated with the indicated volumes of ascetic liquids of monoclonal antibodies 3921E4 (14) anti-HBHA (Figure 5A), or with 250 μ l of rat anti-HBHA polyclonal antiserum (immunity serum, Figure 5B) or naïve serum (Figure 5B, control serum) for 30 minutes, at room temperature. The bacterial
10 suspensions were then washed three times with 2 ml of DPBS to eliminate the non bound antibodies, then used in the CHO cell adhesion test in an infection multiplicity of 10, as described above.

As shown in Figure 5, adhesion of BCG to CHO cells was inhibited substantially in a dose-dependent manner by the presence of anti-HBHA
15 antibodies. The ascites liquids containing the non pertinent monoclonal antibodies or naïve antisera had no effect. These observations indicate that adhesion of mycobacteria to epithelial cells originates in part through the mediation of HBHA.

HBHA characteristics study

20 Since the size of this protein is close to that of fibronectin binding proteins in the antigen 85 complex (15), Western blot analyses were used to determine whether they were related.

125
F8
25 Purified heparin binding protein from a preparation of *M. bovis* BCG cell wall (Figure 3, track 1) or culture supernatant (track 2) was compared with purified antigen 85 complex (track 3) by immunoblot analysis (panels A, B, C) and staining with Coomassie Blue (panel D) after SDS-PAGE. Immunoblot analysis was carried out using polyclonal antibodies directed against the purified heparin binding protein (panel A), the monoclonal antibody 4057D2 (panel B) or polyclonal antibodies directed against antigen complex 85 (panel C). Tracks 1
30 and 2 of panels A, B and C contained 2 μ g of purified protein, tracks 3 of panels

A, B and C contained 7 μ g of purified protein, and tracks 2 and 3 of panel D contained 4 μ g and 15 μ g of purified protein respectively. The size of labels M_r is shown in the margin.

Figure 3 demonstrates that polyclonal antibodies directed against the 28 kDa purified BCG heparin binding protein did not recognise purified antigen 85 complex proteins. In contrast, polyclonal and monoclonal antibodies (not shown) directed against the BCG antigen 85 complex had not succeeded in recognising the heparin binding protein, which implies that the proteins are distinct. This result is also supported by the different migration profiles for these proteins during SDS-PAGE (Figure 3, panel D).

1 The N-terminal amino acids of the purified heparin binding proteins of H37Ra *M. tuberculosis* and of BCG were sequenced. This was accomplished by subjecting 25 μ g of HBHA to polyacrylamide-SDS gel electrophoresis using a 15% polyacrylamide gel. After electrophoresis, the material was transferred to a PVDF membrane (ProBlott, ABI) by electroblotting. After staining with Coomassie blue, the band corresponding to HBHA was excised and underwent automatised Edman degradation. The first 16 amino acids were Ala-Glu-Asn-Ser-Asn-Ile-Asp-Asp-Ile-Lys-Ala-Pro-Leu-Leu-Ala-Ala. The first 16 amino acids in the BCG heparin binding protein were also determined and proved to be identical to those of *M. tuberculosis*. A similarity search in protein databases showed that the heparin binding protein had not been identified before, and that it thus represents a novel mycobacterial protein. Further, the first 16 amino acids did not exhibit any significant sequence similarity with other known protein sequences.

Cloning of the BCG gene coding for HBHA

2 To clone the gene coding for HBHA, firstly the N-terminal sequences of internal HBHA fragments were determined. To this end, purified HBHA underwent electrophoresis as described above. After electrophoresis, the protein was digested with trypsin inside the gel. The resulting peptides were then isolated by reverse phase HPLC, and then underwent Edman degradation. Four peptides enabled the sequence to be determined:

peptide Lys-Ala-Glu-Gly-Tyr-Leu-Glu-Ala-Ala-Thr

S1441:

peptide Xxx-Glu-Gly-Tyr-Val-Asp-Gln-Ala-Val-Glu-Leu-Thr-Gln-Glu-

S1443: Ala-Leu-Gly-Lys

peptide Xxx-Gln-Glu-Xxx-Leu-Pro-Glu-Xxx-Leu

S1446:

peptide Phe-Thr-Ala-Glu-Glu-Leu-Arg

S1447:

1 The sequence of two pairs of oligonucleotides was derived from the
internal HBHA peptide sequences. The generally high G+C content in the
mycobacterial DNA has led the inventors to favour G or C in the third position of
the codons (wobble). The first pair of oligonucleotides originated from the S1441
5 peptide and had the following sequences: 5'AAG GC(G/C) GAG GG(G/C) TAC
CT 3' (oligo S1441) and 5' AGG TA (G/C) CCC TC(G/C) GCC TT 3' (reverse
oligo S1441). The second pair of oligonucleotides originated from the S1443
peptide and had the following sequences: 5'GAC CAG GC(G/C) GT (G/C) GAG
CT 3' (oligo S1443) and 5' AGC TC (G/C) AC(G/C) GCC TGG TC 3' (reverse
10 oligo S1443).

11 The chromosomal BCG DNA was extracted as described by Kremer et al
(23) (16). Polymerisation chain reactions (PCR) using 50 ng of chromosomal
BCG DNA and 1 µg of either reverse S1441 and S1443, or reverse oligo S1443
and S1441, were carried out at a hybridisation temperature of 50°C with 30 PCR
15 cycles. Only the PCR carried out with the reverse S1441 and S1443
oligonucleotides produced a specific amplified DNA fragment of approximately
150 bp. This amplified fragment was again observed when the hybridisation
temperature was increased to 57°C.

20 The amplified fragment was inserted into the *Hind*II site of pUC18
(Boehringer, Mannheim) and introduced into *Escherichia coli* XL1-Blue (New
England Biolabs). The amount of recombinant *E. coli* plasmid was then analysed

using standard methods (17) and the plasmid containing the expected fragment was designated pClone5.

After purification by chromatography on a Nucleobond AX column (Macherey-Nagel) following the instructions of the supplier, a bicatenary DNA
5 fragment of approximately 150 bp was sequenced using the dideoxyribonucleotide chain elongation termination method using [α - 35 S]dCTP (1000 Ci/mole; Amersham) and the T7 sequencing kit (Pharmacia) following the instructions of the manufacturer. The sequence obtained is shown in Figure 7 in which the sequence of two oligonucleotides used for the PCR is underlined. The sequence
10 of amino acids deduced from the DNA sequence was found to correspond to the sequence of amino acids determined for peptides S1441 and S1443. This indicates that the amplified PCR fragment corresponded to an internal portion of the BCG gene coding for HBHA.

In order to clone the whole of the gene coding for HBHA, the 150 bp
15 fragment of pClone5 was excised by digestion with restriction enzymes *Bam*HI and *Hind*II. The fragment was then purified by electrophoresis on a 7% polyacrylamide gel and excised from the gel by electro-elution. The purified fragment was labelled with digoxigenin using the DIG (Boehringer) DNA detection and labelling kit as recommended by the manufacturer. The labelled
20 fragment was then used in Southern Blot experiments to probe the chromosomal BCG DNA digested with *Bam*HI (Figure 8, track 1), *Eco*RI (Figure 8, track 2), *Pst*I (Figure 8, track 3), *Sma*I (Figure 8, track 4), *Acc*I (Figure 8, track 5), *Nco*I (Figure 8, track 6), *Not*I (Figure 8, track 7), *Sac*I (Figure 8, track 8) or *Sph*I (Figure 8, track 9), which underwent electrophoresis on agarose and was
25 transferred to a nylon membrane. The membrane was then probed with the approximately 150 bp *Bam*HI/*Hind*II fragment from pClone5. The marker sizes are shown in the right hand margin. Southern blot analyses were carried out using standard protocols (17). As shown in Figure 8, digestion with chromosomal BCG DNA resulted in a unique fragment of about 2.5 kb which hybridised with the
30 probe.

2.3 to 2.7 kb *SphI* restriction fragments from chromosomal BCG DNA were then purified by preparative electrophoresis and electro-elution, and inserted in the *SphI* site of pUC18. Recombinant plasmids were used to transform *E. coli* XL1-Blue. White colonies cultivated on gelose LB (17) with added ampicillin (150 µg/ml), isopropyl-thiogalactopyrannoside (IPTG, 40 µg/ml) and X-gal (40 µg/ml) were analysed by hybridisation on colonies using the probe labelled with digoxigenin. Of approximately 300 colonies which were analysed, one had hybridised with the probe. Restriction analysis of the plasmid isolated from these clones had indicated that it contained a *SphI* fragment of 2.5 kb which had hybridised with the probe in Southern blot.

Analysis of the sequence of the BCG gene coding for HBHA

The gene coding for HBHA contained in the 2.5 kb *SphI* fragment was sequenced using the dideoxyribonucleotide chain elongation termination method described above. The synthetic oligonucleotide sequences used for sequencing are shown in Table 1, and the sequencing strategy is shown in Figure 9. The cloned 2.5 kb *SphI* fragment is shown by the black line. The dotted lines represent the DNA vector. The bold arrows represent the open reading frame of HBHA. The fine arrows indicate the direction and length of the DNA fragment sequenced for each oligonucleotide indicated above its respective arrow.

TABLE I: Oligonucleotides used for sequencing the gene coding for HBHA.

Name of oligonucleotide	Sequence
HBHA Seq1	5'AGC CGG TAC AAC GAG CTG GTC 3'
HBHA Seq1inv	5'GAC CAG CTC GTT GTA CCG GCT 3'
HBHA Seq2	5'CAT CCA ACA CGT CGA CTC C 3'
HBHA Seq3	5'TTG ATG TCA TCA ATG TTC G 3'
HBHA Seq4	5'CGT GGA CCA GGC GGT GGA G 3'
HBHA Seq5	5'GAC GAT CAG GAG GTT TCC CCG 3'
Reverse primer	5'AGC GGA TAA CAA TTT CAC ACA GGA 3'

The whole of the gene coding for HBHA was localised inside the 2.5 kb *Sph*I fragment at one end of the fragment, and it was completely sequenced from two strands.

The nucleotide sequence and the derived protein sequence are shown in Figure 10. The open reading frame is shown between nucleotides 331 and 927. The first 16 codons after the optional start codon ATG 331/333 are translated into an amino acid sequence which is identical to that determined after N-terminal sequencing of the purified HBHA protein. Upstream of the optional start codon, at residues 331-333, a stop codon TAG is found in phase at positions 310-312. This strongly suggests that the HBHA open reading frame contains no sequences coding for the conventional signal peptide, and 331/333 thus probably represents the start codon. This is supported by the presence of a putative ribosome binding site AGGAA which is found 10 to 6 nucleotides upstream of the start codon.

The invention also concerns any variant of the sequences described above obtained by addition, substitution or deletion of one or more amino acids without substantially modifying the properties of the region of interest. Among the envisaged modifications are silent mutations in the nucleotide sequences which do not modify the peptide sequence of the protein or the fragment of interest, also conservative mutations which consist of substituting one or more amino acids with the same functional characteristics as the amino acid of the native sequence.

Additions or deletions of amino acids without substantially modifying the properties of the regions of interest also form part of the invention. Certain residues can be eliminated or modified by expressing a modified gene in this way in *E. coli* as described for the complete gene. If this protein binds heparin with the same affinity as whole HBHA, this signifies that the repeat sequences are not involved in that binding. In contrast, if the modified protein loses the capacity to interact with heparin, the modified region of the protein plays a role in that interaction.

Expression of the BCG HBHA gene in *Escherichia coli*

The invention also concerns a recombinant host cell, characterized in that it comprises one of the nucleotide sequences described above in its genome. More particularly, the invention concerns the transformation of host cells such as *E. coli* with one of the nucleotide sequences of the invention to produce all or part of the HBHA protein. The bacterial strain to be transformed can comprise the complete sequence coding for HBHA appearing in Figure 10 or the sequence which may be involved in interactions with sulphated glycoconjugates or the C-terminal portion of that sequence. By way of example, the complete gene coding for BCG HBHA was introduced and expressed in *E. coli*.

In order to produce BCG HBHA in *Escherichia coli*, the plasmid derived from pUC18 which contained the 2.5 kb *SphI* fragment comprising the BCG HBHA gene was simultaneously restricted with *NcoI* and *KpnI*. The 705 base pair fragment from this double restriction was purified by electro-elution after migration in a 1% agarose gel using standard procedures (17) and finally cloned in the expression vector pKK388-1 (Clontech, Palo Alto, Ca., UA) previously restricted with *NcoI* and *KpnI*. The recombinant plasmid was then introduced into *E. coli* XL1-Blue using conventional transformation techniques (17).

Phenotype analysis of the strain carrying this expression plasmid was carried out by cultivating it in a liquid LB medium and stimulating the production of recombinant HBHA by adding isopropylthio-galactopyranoside (IPTG) using standard methods (17). Practically, two cultures in 500 ml erlenmeyer flasks each

containing 100 ml of liquid LB medium supplemented with ampicillin in an amount of 150 µg/ml were respectively inoculated with 4 ml of *E. coli* XL1-Blue preculture containing the HBHA expression plasmid or plasmid pKK388-1 representing the control culture. The growth in the two cultures was monitored by measuring the optical density at 600 nm. When this reached 0.6, IPTG was added to the cultures in an amount of 1 mM final and culture was continued for 4 hours. Culture samples were removed before adding the IPTG and at the end of the 4 hours of culture in the presence of an inducer for analysis by polyacrylamide gel electrophoresis and by immunoblotting the production of recombinant HBHA.

The results of these different analyses are shown in Figure 11.

Tracks A and B represent total lysates before introducing *E. coli* XL1-Blue respectively transformed with pKK388-1 and the derivative of pKK388-1 containing the gene coding for HBHA. Tracks C and D represent total lysates after IPTG induction of *E. coli* XL1-Blue respectively transformed with pKK388-1 and the derivative of pKK388-1 containing the gene coding for HBHA. Tracks E contained 1 µg of purified HBHA from BCG walls. From left to right, the different panels respectively represent staining of the polyacrylamide gel with Coomassie blue R-250 and immunoblots probed with a control murine serum, a murine serum directed against BCG HBHA, the murine monoclonal antibody 3921E4 and the murine monoclonal antibody 4057D2. The reference molecular weights are noted to the left of the panel showing staining of the polyacrylamide gel with Coomassie blue.

Analysis of the polyacrylamide gel stained with Coomassie blue shows the synthesis of a polypeptide of about 27 kDa in *E. coli* XL-1 Blue carrying the HBHA expression vector and derepressed by IPTG. This polypeptide, recognised in immunoblotting by a murine antiserum directed against the purified HBHA protein of a BCG culture supernatant, was not produced by *E. coli* XL-1 Blue carrying the pKK388-1 vector with no insert, showing that its synthesis depends on the cloned BCG DNA sequence in the expression vector. As this is also the sequence coding for the BCG HBHA, it was surprising to observe that the

recombinant polypeptide had an apparent molecular weight which was lower than that of the purified BCG HBHA and which was 28 kDa in the gel system under consideration.

125
F-14
5 The invention thus also concerns a recombinant peptide sequence characterized in that it enables mycobacteria to adhere to host cells. More particularly, the invention concerns a peptide sequence comprising a polypeptide of about 25 27 kDa recognised by the monoclonal antibody 3921E4 (14) and not recognised by the monoclonal antibody 4057D2 (14). Preferably, the recombinant peptide sequence of the invention is the expression product of a strain of *E. coli*
10 transformed with one of the nucleotide sequences described above and coding for a peptide sequence enabling mycobacteria to adhere to host cells, more particularly a nucleotide sequence obtained from *M. bovis* BCG or *M. tuberculosis*. The invention also concerns any variation in this recombinant peptide sequence obtained by addition, substitution or deletion of one or more
15 nucleotides such that the transformed strain produces a different peptide sequence but which possesses the property of adhering to host cells, and more particularly to epithelial cells.

Since the molecular weight of the HBHA deduced from the gene sequence is significantly lower than its apparent molecular weight observed after
20 electrophoretic migration and that difference is the result of post-translational modifications of HBHA, these modifications did not occur in *E. coli*. This conclusion is supported by the fact that we observed a differential immunoreactivity for recombinant HBHA with the two monoclonal antibodies 3921E4 and 4057D2. While the two monoclonal antibodies recognise purified BCG HBHA in
25 an equivalent manner on immunoblotting, only 3921E4 was immunoreactive with recombinant HBHA produced in *E. coli*. This observation firstly shows that the epitopes of these two monoclonal antibodies are different and that the epitope recognised by 4057D2 on HBHA is no longer present when the latter is produced in *E. coli*, since it is localised on a molecular element resulting from a post-
30 translational modification not carried out in *E. coli*.

This post-translational modification is a glycosylation since it has already been demonstrated that the saccharide portion of a glycoprotein can be immunogenic. The saccharide moiety is highly immunogenic since immunoblot analysis using anti-HBHA murine serum gave rise to a weaker signal with recombinant HBHA than with that observed with purified BCG HBHA. This observation is all the more telling since the quantity of recombinant HBHA used in this analysis was much larger than that of natural HBHA, as shown by the Coomassie blue staining of the polyacrylamide gel. Further, since the immunoreactivity of 3921E4 was close to that observed with murine serum, the epitope recognised by this monoclonal antibody is thus partially dependent on the suspected post-translational modification.

A number of experimental approaches have been envisaged to elucidate the exact nature of this post-translational modification. Firstly, purified HBHA from BCG is subjected to the action of different endoglycosidases or exoglycosidases in order to see whether the apparent molecular weight of this protein reduces after the action of these enzymes. A more rapid electrophoretic migration observed after digestion with one or more of those enzymes gives direct information about the chemical nature of the saccharide modification. In parallel, BCG cultures in the presence of glycosylation inhibitors (e.g., tunicamycin which inhibits N-glycosylation in gram-positive bacteria) constitute an interesting approach to elucidating the nature of the post-translational modifications of the HBHA.

Identification of the heparin binding site of HBHA

As it has been demonstrated that HBHA plays a direct role in the interaction between BCG and epithelial cells, an interaction which can be inhibited by heparin, it is important to identify the region which is involved in the interaction of BCG with epithelial cells via sulphated polysaccharides.

HBHA produced in the recombinant form by E. coli is the object of proteolysis affecting its carboxyterminal extremity and involving a reduction in heparin

affinity

HBHA produced in *E. coli* WL1 Blue accumulates in the cytoplasm but remains soluble since sonic lysing of colibacilli followed by clarifying centrifugation (10000 g for 15 minutes) placed the whole of the HBHA detectable by immunoblot in the soluble fraction of the lysate. When the latter was
5 incubated at room temperature, the recombinant HBHA degraded rapidly until at the end of an incubation period of 2 hours, its apparent molecular weight had dropped from 27 kDa to 19 kDa. The 19 kDa form which was not recognised by the monoclonal antibody 3921E4 showed, however, good resistance to subsequent degradation since this form remained stable after a period of 18 hours incubation
10 at room temperature. Since the degradation of recombinant HBHA could be stopped by adding 1 mM of native AEBSF which is a serine protease inhibitor, it is probable that this degradation was the result of proteolysis. It should be noted that native HBHA incubated in an *E. coli* XL-1 Blue lysate did not undergo similar degradation, thus suggesting that the post-translational modification in
15 native HBHA endows it with resistance against the proteolysis observed with recombinant HBHA.

Chromatographic analysis on heparin-sepharose of a clarified sonicate of *E. coli* XL-1 Blue producing recombinant HBHA and supplemented with 1 mM of AEBSF showed that recombinant HBHA binds heparin with the same affinity as
20 native HBHA since it eluted at 350 mM of NaCl in PBS. In contrast, HBHA degraded to a 19 kDa polypeptide no longer bound heparin which shows that the cleaved portion is indispensable in recognising sulphated polysaccharides. Recombinant HBHA purified on heparin-sepharose was also shown to be incapable of agglutinating rabbit erythrocytes. These observations show that post-
25 translational modification of HBHA is not involved in its heparin binding activity but it is important for its haemagglutination activity.

Analysis of recombinant HBHA by immobilised heparin matrix chromatography also showed itself to be of interest for mapping the protein domain involved in heparin recognition (the heparin binding site). Three forms of
30 recombinant HBHA with molecular weights of 27, 26 and 25 kDa were eluted by a

linear gradient of NaCl varying from 0 to 500 mM and applied to the washing buffer for the column (PBS). The 26 and 25 kDa forms, degradation products of the complete 27 kDa recombinant protein, eluted at about 310 and 280 mM NaCl respectively. Since the two forms had a low affinity for heparin compared with the complete recombinant protein, it was important to know the region where the truncations occurred. To this end, the amino-terminal extremities of the three purified forms on heparin-sepharose were microsequenced. Analysis of the first 10 amino acids showed that the 3 molecular species had the same sequence which was identical to the amino-terminal sequence of the purified HBHA from BCG walls. This observation thus shows that recombinant HBHA degrades by successive carboxy-terminal cleavages. These cleavages, along with a low affinity for heparin, show that the carboxy-terminal amino acids of HBHA are directly implicated in recognising sulphated polysaccharides. The large amount of lysines in the carboxy-terminal repeat zones of HBHA also argue for an electrostatic interaction between the positive charges on the lysines and the negative charges carried by the sulphated residues of the heparin.

The role of lysines in protein-sulphated sugar interactions has been documented elsewhere (18).

The 19 kDa form observed after proteolysis of recombinant HBHA could thus correspond to an HBHA which has lost its carboxy-terminal repeat sequences corresponding to a loss of 39 amino acids. In this hypothesis, the polypeptide chain of HBHA would lose a weight of about 3.8 kDa and would thus reduce from 21.3 to 17.5 kDa. The apparent molecular weight of recombinant HBHA after proteolysis in an *E. coli* XL-1 Blue lysate is completely compatible with this hypothesis. It is also interesting to note that the aberrant electrophoretic migration of HBHA is attributed to its carboxy-terminal repeat sequences, which is no longer observed with the degraded 19 kDa form which no longer binds heparin. This latter observation thus reinforces the hypothesis that the 19 kDa recombinant HBHA would correspond to an HBHA which has lost its carboxy-terminal repeat sequences. Since the monoclonal 3921E4 antibody no longer recognises the 19

kDa recombinant HBHA, this suggests that its epitope is localised in the carboxy-terminal repeat sequences.

Immune reactivity towards HBHA by antisera from tuberculosis patients

In order to find out whether HBHA is capable of inducing an immune response in man, immuno blot analyses were carried out using purified HBHA and human antisera from tuberculosis patients. Five micrograms of the protein underwent electrophoresis using a 15% polyacrylamide-SDS gel, then were transferred to nitrocellulose membranes subsequently probed with sera diluted 100 fold originating from 7 different patients suffering from evolutive tuberculosis (see Figure 6, tracks 3A at 493) (track 1, purified HBHA, track 2, non pertinent protein), also the serum from a healthy subject (control track). The left hand tracks contained purified proteins (tracks 1 and 2) and the molecular weight markers (see PM) were stained with Coomassie blue after SDS-PAGE.

The results shown in Figure 6 show that all of the serums from tuberculosis patients contained anti-HBHA antibodies, while the serum from a healthy individual contained none of those antibodies. These results indicate that HBHA exposed on the surface is immunogenic during evolution of human tuberculosis and the presence of anti-HBHA antibodies enables the presence of mycobacterial infections to be determined.

The post-translational modification carried by native HBHA is a major antigenic determinant in patients with tuberculosis:

The recombinant HBHA produced by *E. coli* was analysed by immunoblot using the sera from patients with tuberculosis. Compared with the detection signals obtained with native HBHA, these immunological reactants only slightly recognised the recombinant protein, indicating that post-translational modification of the HBHA carried one or more major epitopes. These sera were proved to be incapable of detecting the 19 kDa recombinant HBHA.

The invention thus concerns the use of a peptide sequence or a protein of the invention, in particular one or more immunogenic regions of this sequence or protein, in the diagnosis of mycobacterial infections, in particular by showing the

presence of anti-HBHA antibodies in the biological fluids. In general, a peptide sequence or the protein or a recombinant polypeptide, preferably modified in accordance with the invention, is bonded to a support and incubated with the biological fluids suspected of containing anti-HBHA antibodies. The anti-HBHA antibodies bonded to the polypeptide of the invention are then revealed either, for example, with labelled antibodies directed against the anti-HBHA antibodies, or with non labelled antibodies directed against the anti-HBHA antibodies and antibodies labelled, for example, with a peroxidase or alkaline phosphatase type enzyme, or biotin directed against non labelled antibodies.

In detail, the complete protein or a recombinant polypeptide of the invention can be used but one or more immunogenic regions of that protein can be used which can be determined by epitope mapping techniques which are well known to the skilled person. As an example, to map the B and T epitopes present in the complete HBHA molecule, hydrophobicity profiles are used (19). Computer prediction of antigenic B cell determinants is also used (20).

The polypeptide selected is adsorbed onto a microtitration type plate, canula, microbead or the like. The polypeptide is bound using techniques which are known to the skilled person. Preferably, the support is a microtitration plate. The polypeptide is then diluted in a basic carbonate buffer and placed in the wells. After incubating for several hours at room temperature, a number of washes using a physiological buffer are carried out.

The polypeptide bound to the support is then incubated with a biological fluid sample. Several types of biological fluids can be used, obtained from animals or humans. More particularly, the biological fluid can be obtained from serum, lymph, saliva or urine or it can be isolated from tissues such as cells of the lung. Incubation is carried out using the normal procedures. Here, the sera from patients are diluted and brought into contact with the peptide sequence bound to the plate. Incubation of about one hour is followed by several washes with a physiological buffer. The anti-HBHA antibodies bonded to the polypeptide of the invention are then revealed either with the labelled antibodies directed against the

anti-HBHA antibodies, or with non labelled antibodies directed against the anti-HBHA antibodies then with labelled antibodies directed against the non labelled antibodies.

5 The antibodies can be labelled radioactively but in general, a further normal type of labelling is preferred. Fluorescent substances such as esithiocyanatefluoriscein or enzymes such as alkaline phosphatase, peroxidase or biotin/streptavidin are routinely used labels. The choice of labelled on non labelled antibodies depends on the animal from which the biological fluids originate. If the biological fluid is human, the antibodies used are directed against
10 human immunoglobulins.

Binding between the anti-HBHA antibodies and the labelled or non labelled antibodies is carried out using the normal techniques. As an example, the reaction takes place over one hour at room temperature and after several washes, a labelling substrate is added.

15 The invention also provides a kit for detecting the presence of anti-HBHA antibodies in a sample of a biological fluid. The kit comprises a polypeptide, or one or more imunogenic regions of the HBHA, adsorbed on a support and optionally, a labelled antibody (and if necessary a non labelled antibody) as well as the normal buffers and a substrate for the label.

BIBLIOGRAPHY

1. Smith, P.G., & Moss, A.R. (1994) in *Tuberculosis, Pathogenesis, Protection, and Control*, ed. Bloom, B.R. (ASM Press, Washington, DC), pp.47-59.
- 5 2. Raviglione, M.C., Snider, D.E., & Kochi, A. (1995) *J. Am. Med. Assoc.* 273, 220-226.
3. Noordeen, S.K., Bravo, L.L., & Sundaresan, T.K. (1992) *Bull. WHO* 70, 7.
4. Horsburgh, C.R. (1991) *N. Engl. J. Med.* 324, 1332.
5. Barnes, P.F., Bloch, A.B., Davidson, P.T., & Snider, D.E. (1991) *N. Engl. J.*
10 *Med.* 324, 1644.
6. Bloch, A.B., Cauthen, G.M., Onorato, I.M., Dansbury, K.G., Kelly, G.D.,
Driver, C.R., & Snider, D.E. (1994) *J. Am. Med. Assoc.* 271, 665-671.
7. Bloom, B.R., & Murray, C.L. (1992) *Science* 257, 1005-1064.
8. Riley, L.W. (1995) *Trends Microbiol.* 3, 27-31.
- 15 9. Shepard, C.C. (1957) *J Exp. Med.* 105, 39-48.
10. Schlesinger, L.S., Bellinger-Kawahara, C.G., Payne, N.R., & Horwitz, M. A.
(1990) *J. Immunol.* 144, 2771-2750.
11. Schlesinger, L.S., & Horwitz, M.A. (1990) *J. Clin. Invest.* 85, 1304-1314.
12. Hirsch, C.S., Ellner, J.J., Russell, D.G., & Rich, E.A. (1994) *J. Immunol.* 152,
20 743-753.
13. Laemmli, U.K. (1970) *Nature (London)* 227, 680-685.
14. Rouse, D.A., Morris, S.L., Karpus, A.B., Mackall, J.C., Probst, P.G., &
Chaparas, S.D. (1991) *Infect. Immun.* 59, 2595-2600.
15. Wiker, H.G., & Harboe, M. (1992) *Microbiol Rev.* 56, 648-661.
- 25 16. Kremer, L., Baulard, A., Estaquier, J., Poulain-Godefroy, O., & Locht, C.
(1995) *Mol. Microbiol* 17, 913-922.
17. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning. A
Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor
Laboratory Press.
- 30 18. A.D. CARDIN & H.J.R. WEINTRAUB, 1989, Molecular modelling of

protein glycoaminoglycans interactions, Arteriosclerosis, 9, 21-32.

19. T.P. Hopp, 1989, Use of hydrophilicity plotting procedures to identify protein antigenic segments and other interaction sites, Methods in Enzymology, 178, 571-585.
- 5 20. V. Krchnak, O. Mach and A. Maly, 1989, Computer prediction of B-cell determinants from protein amino acid sequences based on incidence of β turns, Methods in Enzymology, 178, 586-611.